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The Adenovirus E4-6/7 Protein Transactivates the E2 Promoter by Inducing Dimerization of a Heteromeric E2F Complex

SABINE OBERT, ROBERT J. O'CONNOR, SUSANNE SCHMID, AND PATRICK HEARING*

Department of Microbiology, Health Sciences Center, State University of New York, Stony Brook, New York 11794

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Binding of the mammalian transcription factor E2F to the adenovirus E2a early promoter is modulated through interaction with the viral E4-6/7 protein. E4-6/7 induces the cooperative and stable binding of E2F in vitro to two correctly spaced and inverted E2F binding sites in the E2a promoter (E2F induction) by physical interaction in the protein-DNA complex. The E2a promoter is transactivated in vivo by the E4-6/7 product. The C-terminal 70 amino acids of E4-6/7 are necessary and sufficient for induction of E2F binding and for transactivation. To assess the mechanism(s) of E2a transactivation and the induction of cooperative E2F binding by the E4-6/7 protein, we have analyzed a series of point mutants in the functional C-terminal domain of E4-6/7. Two distinct segments of E4-6/7 are required for interaction with E2F. Additionally, an E4-6/7 mutant with a phenylalanine-to-proline substitution at amino acid 125 (F-125-P) efficiently interacts with E2F but does not induce E2F binding to the E2a promoter and is defective for transactivation. Induction of E2F stable complex formation at the E2a promoter by the F-125-P mutant protein is restored by divalent E4-6/7-specific monoclonal antibodies, but not a monovalent Fab fragment, or by appending a heterologous dimerization domain to the N terminus of the mutant protein. These and other data support the involvement of E4-6/7 dimerization in the induction of cooperative and stable E2F binding and transactivation of the E2a promoter. We present evidence that at least two cellular components are involved in E2F DNA binding activity and that both are required for E2F induction by the E4-6/7 product. The recently cloned E2F-related activities E2F-1 and DP-1 individually bind to an E2F binding site weakly, but when combined generate an activity that is indistinguishable from endogenous cellular E2F. Recombinant E2F-1, DP-1, and E4-6/7 are sufficient to form the induced E2F complex at the E2a promoter.

Modulation of transcription factor activity by protein-protein interaction has emerged as a theme in transcriptional regulation. The mammalian transcription factor E2F was first described as a nuclear activity that binds to the adenovirus (Ad) E2 promoter (30, 31). Functional E2F recognition sites are present in the promoter regions of cellular genes such as c-*myc* and dihydrofolate reductase (7, 26, 39, 57). Recent studies have focused attention on the protein contacts of E2F (reviewed in reference 43). The association of E2F with cellular proteins varies with specific stages of the cell cycle. A protein complex containing E2F, p107, cyclin A, and kinase cdk2 is detected during S phase, while p107, cyclin E, and cdk2 are components of an E2F complex during G_1 phase (8, 14, 35, 38, 46, 52). The p107 protein appears to contact E2F directly and mediate association with cyclins and kinase. The product of the retinoblastoma tumor suppressor gene (Rb) is another direct binding partner of E2F (2, 5, 10, 11). Inhibition of cell proliferation, a hallmark of normal Rb function, is relieved when the Rb-E2F interaction is lost by mutation of Rb or dissociated by the DNA tumor virus transforming proteins T antigen, E1A, or E7 (9, 25, 48, 60, 64). This implicates E2F as a target in Rb signaling pathways and transcriptional regulation linked to E2F as one of the molecular mechanisms by which Rb functions.

The regulation of the E2F DNA binding activity by protein interactions will not be explained without a definition of E2F in terms of its protein components. Recently, an Rb-binding protein with the characteristics of E2F was cloned (23, 29, 51). This protein, referred to as E2F-1, is distinct from DP-1, whose cDNA was isolated following purification of an F9 cell E2F-like activity (16). E2F-1 contains a potential basic helix-loop-helix region with an adjacent leucine zipper (29), and shows significant similarity to DP-1 in helix II and the zipper region (16). Another recent report described the sequence-specific DNA affinity purification of multiple E2F activities (28). Five proteins that belong to two functional groups were isolated; heterodimerization of one of these E2F components with a member of the other group is required to restore optimal DNA and Rb binding. Proteins in group I cross-react with recombinant E2F-1-specific antiserum, while proteins in group II interact with E2F-1 (28). E2F activity, therefore, likely consists of a new family of related proteins with the ability to dimerize and to form multiple protein complexes. The differential regulation of target genes may depend on the binding affinities of each member of the family for a given promoter, on the regulation of this affinity by protein contacts within the family and/or with other proteins not contacting DNA, and on other factors binding to different sites in the upstream regulatory region.

During Ad infection, E2F binding to the well-characterized Ad E2a early promoter is modulated through direct interaction with a viral protein. The E2a early promoter contains a TATA-like motif at positions −22 to −30, two E2F binding sites in an inverted repeat orientation between nucleotides −36 to −43 and −60 to −67, and an ATF binding site at −69 to −79 (40, 53, 62, 63). In vitro binding of E2F to the E2a promoter is dramatically induced in nuclear extracts from Ad-infected cells compared with extracts from uninfected cells (termed E2F induction) (30, 31). This induction

* Corresponding author. Phone: (516) 632-8813. Fax: (516) 632-8891.

results from cooperative and stable binding of E2F to the correctly spaced and oriented E2F sites (21, 49). The viral product responsible for the generation of the infection-specific form of E2F is the 150-amino-acid E4-6/7 protein (27, 37, 41, 49). E4-6/7 is physically present in the cooperative, stable protein-DNA complex at the E2a promoter (double-site complex) and also forms a complex with E2F and a single E2F binding site (single-site complex) (27, 37, 41, 49). The significance of these complexes is not clear in terms of transcriptional function. When expression from the E2a promoter was analyzed in vivo in HeLa cells infected with an E4-expressing virus, both E2F sites, the ATF site, and the TATA-like element were found to be necessary for efficient basal transcription and optimal transactivation by Ad E1A (36). The Ad E1A proteins are not required for induction of E2F binding in vitro (27, 37, 41, 42, 44). The E4-6/7 protein is able to transactivate the E2a promoter in transient transfections (42, 44), and the presence of the E4-6/7 coding region in the virus is essential for maximum accumulation of E2 mRNA in certain cells (37).

It is not known how the physical interaction of E4-6/7 with E2F or formation of the ternary protein-DNA complexes is related to transcriptional stimulation. The possibility of a linkage between the mechanism of E4-6/7 transactivation of the E2a promoter and the mechanism of induction of cooperative E2F binding to this region has prompted us to analyze E4-6/7 genetically in order to assess the nature of its interaction with E2F. Analyses with deletion mutants in E4-6/7 defined the C-terminal 70 amino acids as necessary and sufficient for induction of cooperative binding and for transactivation (42, 44). Here we describe a point mutation in E4-6/7 (at amino acid 125) that does not diminish the ability of the mutant protein to interact with E2F at a single site but that abolishes induction of double-site E2F binding in vitro and transactivation of the E2a promoter in vivo. Induction of stable E2F binding to the E2a promoter can be restored to the mutant protein by a divalent, but not monovalent, anti-E4-6/7 monoclonal antibody or by appending a heterologous protein dimerization motif to the N terminus of the mutant product. These results indicate the involvement of E4-6/7 dimerization in the induction of cooperative E2F binding and E2a promoter transactivation. We also present evidence that at least two cellular components are involved in E2F binding at a single E2F site, and in the formation of the double-site complex at the E2a promoter with the E4-6/7 product. The recently cloned activities E2F-1 and DP-1 (16, 23, 29, 51) in addition to the E4-6/7 protein are sufficient to form the double-site complex at the E2a promoter.

MATERIALS AND METHODS

Bacterial and in vitro expression of proteins. The bacterial expression plasmid pET-3c-6/7 was constructed with the pET-3 vector system described by Dunn and Studier (50). The Ad type 2 (Ad2) E4-6/7 cDNA (58) was excised from pBS-E4-6/7 (44) with *Tth*111I and *Cla*I and blunt end ligated into the *Bam*HI cloning site of the pET-3c vector. The two N-terminal amino acids of E4-6/7 are substituted by 13 amino acids of the T7 gene 10 protein. E4-6/7 point mutations were generated in the Ad2 E4-6/7 cDNA by the method of Kunkel (33) in a Bluescript vector and transferred into the pET-3c-6/7 vector. BL21(DE3) lysogen (56) transformed with pET-3c-6/7 was grown to an optical density (600 nm) of 0.8 to 1.0, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM, and incubation was continued for 2 h. Bacterial pellets were harvested by centrifugation at 8,000 × *g* for 5 min at 4°C and resuspended in 1/50 the original volume of TNE (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) and 0.5 mM phenylmethylsulfonyl fluoride. Bacteria were lysed by incubation with lysozyme (1 mg/ml) and deoxycholic acid to 0.1% and subsequently digested with DNase I. Inclusion bodies containing the overexpressed E4-6/7 proteins were washed with TNE plus phenylmethylsulfonyl fluoride and resuspended in 8 M urea in TNE containing 15 mM dithiothreitol (DTT). Remaining particulate material was removed by centrifugation. E4-6/7 proteins were visualized and standardized by Coomassie blue-stained sodium dodecyl sulfate (SDS)–20% polyacrylamide gel electrophoresis (PAGE). The Ad2 E4-6/7 cDNA (amino acids 2 to 150, *Tth*111I-*Hin*dIII) was excised from pBS-E4-6/7 and inserted into the glutathione *S*-transferase vector pGEX-2T (18) between the *Eco*RI and *Hin*dIII sites. The ZIP-E4-6/7 hybrid proteins contain E4-6/7 amino acids 1 and 2 fused to the leucine zipper of C/EBP (34) (amino acids 297 to 354), which was fused to amino acids 70 to 150 of wild-type (WT) E4-6/7 and the F-125-P mutant in a Bluescript vector.

The E2F-1 cDNA (23) was kindly provided by K. Helin and E. Harlow. The E2F-1 reading frame (amino acids 1 to 347) was inserted in the pET-3a vector and expressed as described above. The DP-1 cDNA (16) was kindly provided by R. Girling and N. LaThangue in vector pGC (59).

Plasmids were linearized and transcribed in vitro with T7 RNA polymerase. Wheat germ extracts and reticulocyte lysates were programmed with 1 to 2 μg of RNA in the presence of ^{35}S-Translabel as described by the manufacturer (Promega). In vitro translation products were normalized within experiments by trichloroacetic acid precipitation and analyzed by SDS-PAGE and autoradiography.

Extract preparation and gel mobility shift assays. Nuclear and cytoplasmic extracts were prepared according to the method of Dignam and Roeder (15). The supernatant obtained after isolation of the nuclei was adjusted to 100 mM KCl, spun at 100,000 × *g* for 1 h, and saved as the cytoplasmic fraction. Cytoplasmic and nuclear fractions were dialyzed against DB (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 100 mM KCl, 10% glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), and the dialysate was cleared by centrifugation at 25,000 × *g*.

In vitro DNA binding assays were essentially performed as described previously (44). Briefly, binding reaction mixtures (20 μl) contained 5 to 10 μg of nuclear or cytoplasmic extract, 2 μg of sonicated salmon sperm DNA, and 20,000 cpm (double-site probe) or 40,000 cpm (single-site probe) of ^{32}P-labeled E2F recognition sites (1 to 2 fmol of DNA) in DB supplemented with Nonidet P-40 (final concentration, 0.1%). In binding reaction mixtures involving urea-solubilized E4-6/7, the final DTT concentration was 5 mM. Urea-solubilized E4-6/7 proteins, normalized for protein concentration by SDS-PAGE, were diluted serially into DB containing 15 mM DTT to a final dilution of 10,000-fold in the binding reaction mixtures. Binding reaction mixtures containing bacterially expressed E4-6/7 were incubated overnight at 4°C before electrophoresis on a 4% polyacrylamide gel. Other binding reactions were performed for 2 h at room temperature. The E2F double-site probe contains nucleotides −30 to −73 from the E2a promoter plus additional vector sequences. The sequence of one strand of the double-site probe and the competitor oligonucleotide is 5′-*AATT*CGTAGTTTCGCG CTTAAATTTGAGAAAGGGCGCGAAACTAGTC*CC GG*-3′; E2F sites are underlined, and vector sequences are

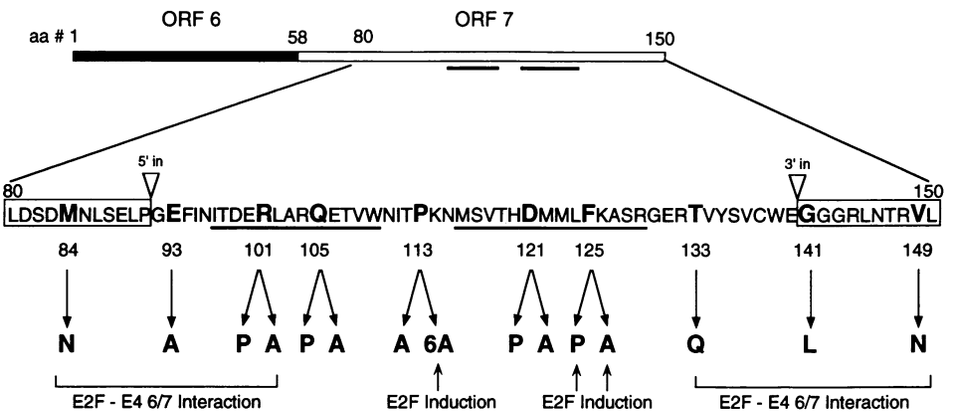


FIG. 1. Mutations in the E4-6/7 C-terminal domain. The top of the schematic depicts the reading frame of the 150-amino-acid E4-6/7 protein. Amino acids 1 to 58 are derived from E4-ORF6 while amino acids 59 to 150 are derived from E4-ORF7. The positions of two putative α helices described in the text are indicated by boldface bars below the E4-6/7 reading frame. Shown below is the amino acid sequence of the C-terminal 70 amino acids of the E4-6/7 protein. The positions of the putative α helices are underlined. Boxed amino acids indicate the positions of deletion mutations previously described (44). 5′ in and 3′ in indicate the positions of four-amino-acid insertion mutations previously described (5′ in [44]) or described in the text (3′ in). Amino acids indicated in large boldface represent the sites of amino acid substitutions. The amino acid number of each mutation is indicated below the sequence as is the specific substitution present at each location. The regions of the E4-6/7 protein that are involved in interaction with E2F are indicated by brackets at the bottom, and the specific mutations that decrease E2F induction are indicated by arrows.

shown in italics. The E2F single-site probe contains Ad nucleotides 270 to 293 from the E1A enhancer and flanking vector sequences: 5′-*AATTCCC*CCATTTTCGCGGGAAA ACTGAAT*CCTCGA*-3′. Probe fragments were labeled with [α-^{32}P]dATP and Klenow polymerase, separated from vector DNA by electroelution from gel slices, and precipitated with ethanol. Specific activities were 5,000 to 10,000 cpm/fmol.

Viruses, infections, and transactivation assay. Monolayer HeLa cells were maintained in Dulbecco's modified minimal essential medium containing 10% calf serum (HyClone). Suspension cultures of HeLa cells were grown in minimal essential medium containing 7% calf serum. Ad5 *wt*300 and *dl*356 viruses were described previously (20). *dl*356 contains a 2-bp deletion in E4-ORF7 which disrupts the reading frame downstream of amino acid 91 and leads to expression of a truncated, nonfunctional E4-6/7 product. Ad-CMV-6/7-WT and Ad-CMV-6/7-F125P were constructed by inserting the Ad2 WT 6/7 or 6/7-F125P coding sequence under the control of the cytomegalovirus promoter/enhancer (55) into the E1A region (deletion of Ad5 nucleotides 355 to 811) of *dl*356. The E2-LS-CAT virus contains the *Xho*I-to-*Xba*I fragment of pE2a-E-CAT-LS-74/-85 (40) in place of the E1A region (nucleotides 355 to 811) of *dl*356.

For transactivation assays, monolayer HeLa cells were grown to 75% confluency. Cells were infected with E2-LS-CAT at 100 particles per cell and coinfected with Ad5 *wt*300, *dl*356, Ad-CMV-6/7-WT, or Ad-CMV-6/7-F125P at 50 particles per cell (2 to 4 PFU per cell). At 16 h postinfection, cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (17). Acetylated and nonacetylated forms of [^{14}C]chloramphenicol were excised from the thin-layer silica plate and quantitated by liquid scintillation spectroscopy. HeLa suspension cells were infected at 200 particles per cell (approximately 5 to 10 PFU per cell) and harvested 6 h postinfection for nuclear extract preparation. To generate metabolically labeled extracts, monolayer HeLa cells were infected at 1,000 particles per cell (50 PFU per cell). At 4 h postinfection, cells were labeled in Dulbecco's modified minimal essential medium lacking methionine with 300 μCi of ^{35}S-Translabel per 5×10^6 cells. Cells were harvested 2 h later for nuclear extract preparation.

Antibodies, Western blot (immunoblot) analysis, and immunoprecipitations. Monoclonal antibodies (MAbs) M45, M41, and M80 were raised in BALB/c mice against insoluble bacterially expressed WT E4-6/7 protein by standard procedures. Hybridoma supernatants were initially screened by enzyme-linked immunosorbent assay. Positive hybridomas were cloned at least twice by limited dilution and screened by immunoprecipitation of ^{35}S-labeled E4-6/7 from reticulocyte lysate translations. Antibodies were concentrated and purified from hybridoma supernatants on protein A-Sepharose beads (Pharmacia) as described by Harlow and Lane (22). Fab fragments were generated as described by Parham (47). Briefly, 800-μl papain digestion reaction mixtures contained 2 to 4 mg of immunoglobulin G2a in digestion buffer (150 mM NaCl, 10 mM phosphate [pH 6.7]), 5 mM fresh cysteine-free base (Sigma), and 1 mM EDTA. A total of 0.2 μg of papain (crystalline suspension; Worthington) was added as empirically determined for 30 min at 37°C. The reaction was stopped by alkylation with iodoacetamide (final concentration, 7.5 mM) for 30 min at room temperature. The reaction mixture was cleared at 10,000 × *g* for 30 min and passed over a protein A-Sepharose column at 0.3 ml/min to separate Fab fragments in the flowthrough from Fc and undigested immunoglobulin G. Digestion and purification were monitored by SDS-PAGE and silver staining. Protein A-purified M41 was concentrated 5- to 10-fold on a Centricon microconcentrator (Amicon) to a final concentration of ~0.5 to 1 μg/μl as estimated by silver staining.

For Western blot analysis of E4-6/7 expressed in the transactivation assay, cell pellets from one plate (6×10^6 cells) of each coinfection were boiled in 2× sample buffer (2% SDS, 1/10 [vol/vol] beta-mercaptoethanol), diluted back to a final concentration of 0.1% SDS, and immunoprecipitated with M80. The precipitate was washed once with radioimmunoprecipitation assay buffer (RIPA), released by boiling in 2× sample buffer, and run on SDS–20% PAGE. Proteins were transferred to nitrocellulose and probed with

M80. Signals were detected by secondary horseradish peroxidase-conjugated antibody and enhanced chemiluminescence as recommended by the manufacturer (Amersham). For the E4-6/7 and E2F solution binding assay, 10× to 20× binding reaction mixtures without probe were incubated on ice for 1 h. M45 was added, and incubation was continued on ice for an additional hour. Immunoprecipitates were collected on protein A-Sepharose and washed for 5 min each with RIPA buffer (1% Triton, 1% Na deoxycholate, 0.1% SDS, 160 mM NaCl, 10 mM Tris-HCl [pH 7.4], 5 mM EDTA), high-salt buffer {1 M NaCl, 10 mM Tris-HCl [pH 7.4], 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]}, RIPA buffer, and twice with TEN (50 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA). Finally, precipitated proteins were released from the antibody by overnight incubation in DB containing 0.1% Nonidet P-40 and P2 peptide. After the beads were spun out, the supernatant (P2 eluate) was assayed by addition of ^{32}P-labeled probe for in vitro binding or analyzed by SDS-PAGE. P2 peptide (SRDRLPPFETETGGY) has been described previously (12). It contains amino acids 26 to 37 of E4-ORF6 and blocks binding of M45 in a mobility shift assay.

E2F gel slicing experiment. E2F activity in the heparin-agarose fraction of HeLa cell E2F (61) was boiled in 2× sample buffer and fractionated on an SDS–10% polyacrylamide gel. Proteins corresponding to different molecular masses between ~40 and ~70 kDa were eluted from gel slices by the procedure of Hager and Burgess (19). Following precipitation with acetone, the eluted proteins were resuspended in DB containing 8 M urea. E2F and associated activities were assayed by direct dilution into DNA binding reaction mixtures.

RESULTS

The ability of E4-6/7 to induce E2F binding to a double site can be separated from its interaction with E2F at a single binding site. The C-terminal 70 amino acids of the E4-6/7 protein are essential and sufficient for interaction with E2F at a single binding site and for induction of cooperative binding to the double site in the E2a promoter (42, 44). In order to further define structural elements of E4-6/7, we replaced single amino acids at regular intervals in this C-terminal functional domain (Fig. 1). Computer analysis predicts the existence of two α helices located between amino acids 97 and 109 and acids 116 and 129, separated by a short six-amino-acid spacer region with a proline at position 113. We chose to substitute two amino acids within each putative α helix with either alanine (R-101-A, Q-105-A, D-121-A, F-125-A), which is not expected to affect the overall structure, or proline (R-101-P, Q-105-P, D-121-P, F-125-P), which is considered a helix breaker (6). The proline in the spacer region was replaced by an alanine (P-113-A) or flanked by a six-amino-acid insertion (AAA PAAA). In addition, every eighth amino acid outside the putative α helices was substituted with relatively nonconservative amino acids (M-84-N, E-93-A, T-133-Q, G-141-L, V-149-N). The mutants were produced in the context of the full-length E4-6/7 protein in a T7 bacterial expression system and tested for reconstitution of the infection-specific E2F complexes by gel mobility shift assays. Recombinant denatured E4-6/7 proteins were renatured and added to in vitro DNA binding reaction mixtures containing cytoplasmic extract from *dl*356-infected HeLa cells as a source of E2F (Fig. 2). The presence of E1A in these E4-6/7-negative extracts disrupts complexes of E2F with cellular proteins (1) to allow unambiguous interpretation of E4 mutants that show a reduced induction of E2F DNA binding. Experiments using nuclear extract from *dl*356-infected cells gave identical results (data not shown).

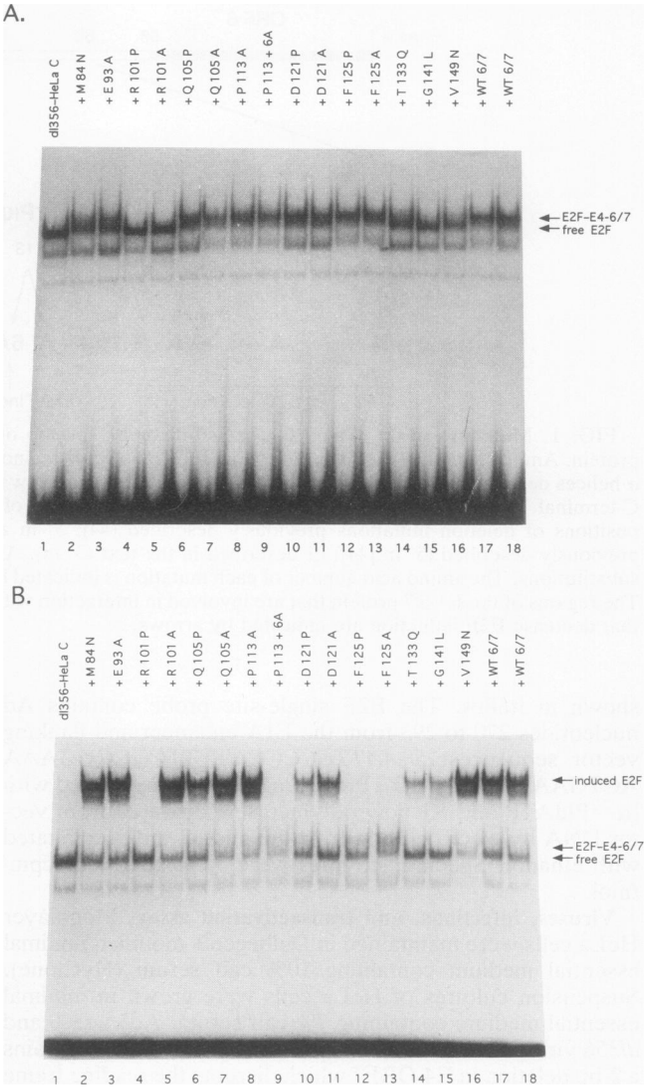


FIG. 2. (A) Interaction of E4-6/7 with E2F at a single E2F binding site. A DNA probe containing a single E2F binding site was incubated with cytoplasmic extract from *dl*356-infected HeLa cells alone (lane 1) or with WT E4-6/7 (lanes 17 and 18) or mutant E4-6/7 proteins (lanes 2 to 16). Protein-DNA complexes were resolved by gel mobility shift assay. The specific E4-6/7 mutations are indicated above each lane. The positions of free E2F and the E2F–E4-6/7 complex are indicated by arrows at the right. The protein-DNA complex migrating faster than the free E2F complex represents a free form of E2F that only weakly interacts with the E4-6/7 protein. The fastest-migrating complex is nonspecific. (B) Interaction of E4-6/7 with E2F at a double E2F binding site. A DNA probe containing the double E2F binding site from the E2a promoter was incubated with cytoplasmic extract from *dl*356-infected HeLa cells alone (lane 1) or with WT E4-6/7 (lanes 17 and 18) or mutant E4-6/7 proteins (lanes 2 to 16), and protein-DNA complexes were resolved by gel mobility shift assay. The specific E4-6/7 mutations are indicated above the lanes. The positions of free E2F, the E2F–E4-6/7 single-site complex, and the induced E2F double-site complex are indicated at the right.

The interaction of the E4-6/7 mutants with E2F was tested with a labeled DNA fragment from the Ad E1A enhancer region that contains a single E2F binding site (Fig. 2A). In the absence of functional E4-6/7 protein, E2F generates two characteristic complexes with this probe (lane 1). The weak complex with the fastest mobility seen in all lanes is nonspecific. WT E4-6/7 protein forms a complex with E2F on the single site and retards the mobility of the upper E2F band (lanes 17 and 18). Mutation of R-101 to P or A dramatically reduced E2F–E4-6/7 single-site complex formation (lanes 4 and 5). Interaction with E2F at the single site was also greatly decreased by mutation of amino acids G-141 and V-149 (lanes 15 and 16) and detectably altered with mutants M-84-N, E-93-A, and T-133-Q (lanes 2, 3, and 14). The other point mutations did not reduce the interaction of E4-6/7 with E2F at the single site. A labeled DNA fragment containing the two inverted E2F sites from the E2a promoter was used to test the variant E4-6/7 proteins for their ability to induce E2F binding at the double site (Fig. 2B). Induction of E2F activity by the WT E4-6/7 protein is shown in lanes 17 and 18 versus lane 1. F-125 is a critical residue for E2F induction in this assay. The F-125-P and F-125-A mutant proteins were defective for E2F induction at the double site and instead formed a complex with the same mobility as that of WT E4-6/7 protein binding to E2F at a single E2F site (lanes 12 and 13). Insertion of six amino acids into the spacer region between the putative α helices also decreased the quantity of the double-site complex (P-113 + 6A, lane 9), while a change of P-113 to alanine did not have a pronounced effect (P-113-A, lane 8). Two other insertion mutants (5′ in [data not shown] and 3′ in [44] [Fig. 1]) also were greatly diminished for induction of E2F binding at a double site; they also were impaired for interaction with E2F at a single site. Proline substitution at two other positions within the putative α helices did not significantly reduce induction of E2F binding at the double site (Q-105-P and D-121-P, lanes 6 and 10) whereas a change of R-101 to proline moderately reduced formation of the double-site complex. The other mutant proteins induced E2F binding to the double site at levels that were comparable to that for the wild-type product; we note that two- to threefold differences in induction levels between the WT and mutant proteins are not considered significant and represent the variations between assays. When the individual mutant proteins were produced in vitro with reticulocyte lysates or wheat germ translation extracts (in the context of a C-terminal 90-amino-acid product), their phenotypes in the single-site and double-site E2F binding and induction assays were identical to the results obtained with the bacterially produced proteins, with the exception of F-125-A, which showed weak induction of E2F binding to the double site (data not shown).

These data demonstrate that two segments of the E4-6/7 protein are involved in interaction with E2F (Fig. 1, brackets below sequence) and that interaction with E2F at a single binding site can be uncoupled from induction of E2F binding to the double site in the E2a promoter. These results also show that the interaction of E4-6/7 with E2F in the presence of the double site (mutants F-125-P and F-125-A) is not sufficient to induce cooperative and stable binding of E2F. Comparison of the binding patterns of proline and alanine substitution mutants (Q-150-P versus Q-105-A, D-121-P versus D-121-A, and F-125-P versus F-125-A) does not support a correlation between the putative α helices and either E2F interaction or induction of E2F binding.

Interaction with E2F is not sufficient for E4-6/7 transactivation of the E2 promoter. To assay the transactivation

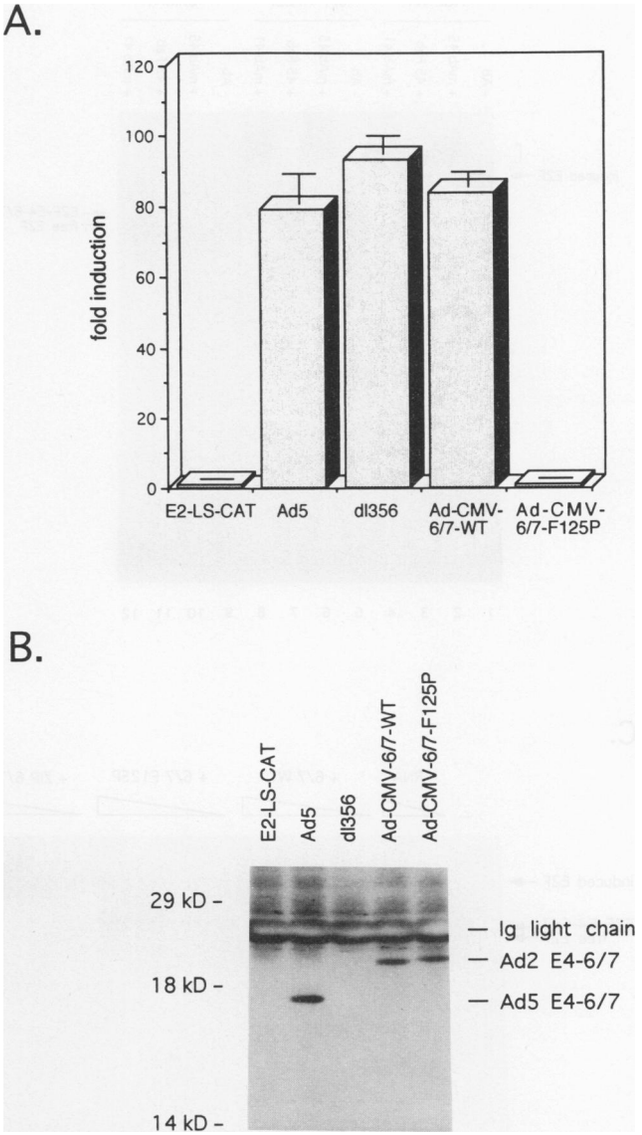


FIG. 3. Transactivation of the E2a promoter by E4-6/7. (A) HeLa cells were infected with a recombinant Ad carrying the E2a promoter fused to the CAT gene (E2-LS-CAT) or coinfected with the E2-CAT virus and WT Ad5 (Ad5), a virus that expresses E1A but not the E4-6/7 protein (*dl*356), a virus that expresses the WT E4-6/7 protein but not E1A (Ad-CMV-6/7-WT), or a virus that expresses the E4-6/7-F125P mutant protein but not E1A (Ad-CMV-6/7-F125P). Cellular extracts were prepared 16 h after infection and assayed for CAT activity. The level of expression of the E2-CAT virus alone is set at 1. The relative levels of expression with the different coinfections are indicated as fold induction. The results represent the average of three independent experiments, and standard deviations are shown for the data. (B) An aliquot of each cellular extract prepared following infection of HeLa cells as described for panel A was immunoprecipitated with M80 and fractionated by SDS-PAGE. E4-6/7 proteins were detected by Western blot analysis with M80. The positions of the Ad5 E4-6/7 protein, the WT and F-125-P mutant Ad2 E4-6/7 proteins, and the immunoglobulin light chains (from the immunoprecipitation and illuminated by the second antibody) are indicated on the right. The positions of molecular mass markers are indicated at the left.

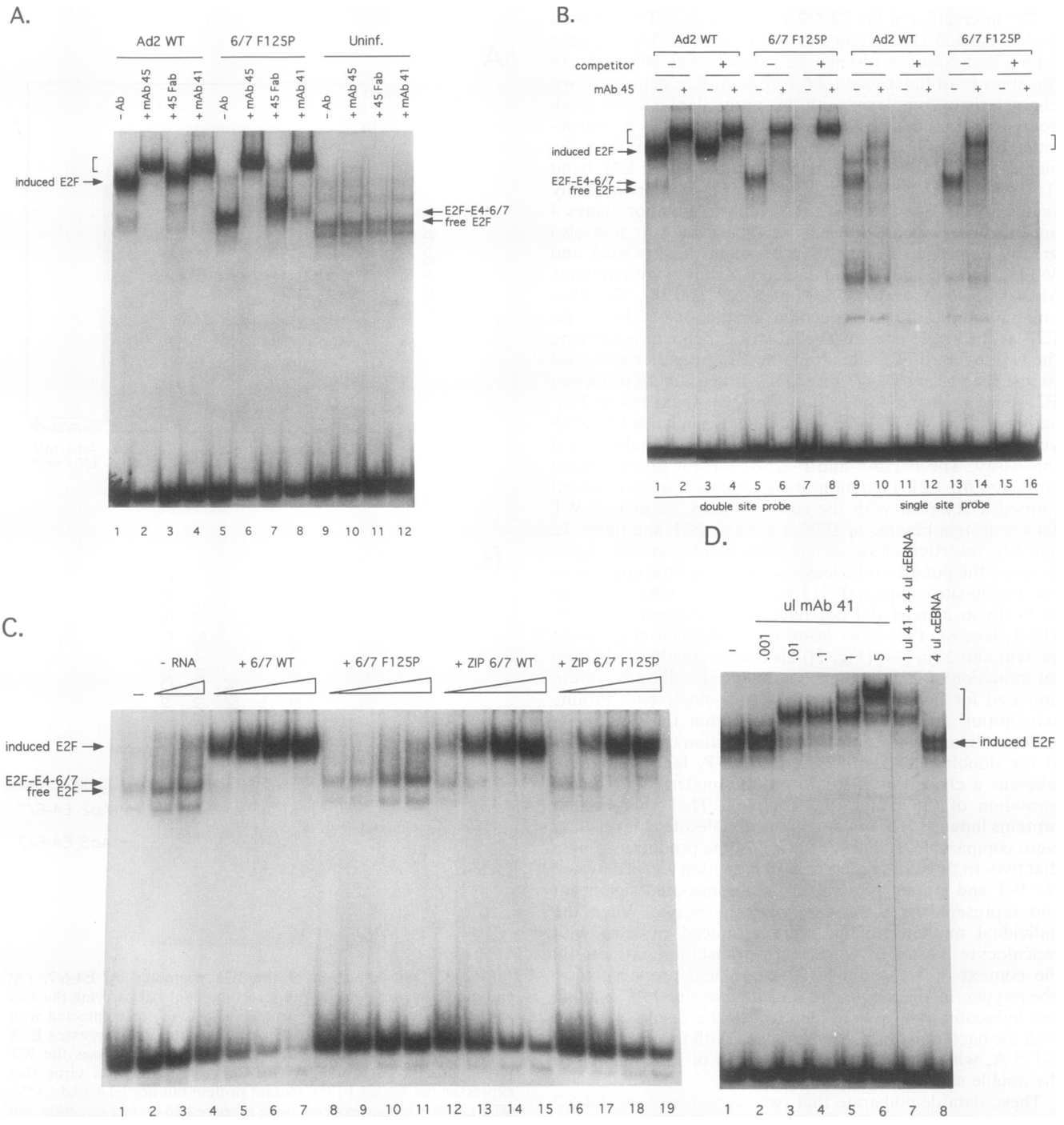


FIG. 4. The E4-6/7 protein appears to induce E2F binding by dimerization. (A) Nuclear extracts were prepared from uninfected HeLa cells (lanes 9 to 12) or from HeLa cells infected with a virus that expresses the WT E4-6/7 protein (lanes 1 to 4) or the F-125-P mutant E4-6/7 protein (lanes 5 to 8). The extracts were incubated in binding reaction mixtures with a DNA probe containing the E2F double site from the E2a promoter, and protein-DNA complexes were resolved by gel mobility shift assay. Lanes 1, 5, and 9 represent binding reactions with extract alone. Lanes 2, 6, and 10 represent binding reactions in which M45 (mAb45) was incubated in the reaction mixture. Lanes 3, 7, and 11 are binding reactions in which an Fab fragment of M45 was incubated in the reaction mixture. Lanes 4, 8, and 12 are binding reactions in which M41 (mAb41) was incubated in the reaction mixture. The positions of free E2F and the E2F–E4-6/7 single-site complex are indicated at the right. The position of induced E2F is indicated at the left, and the positions of complexes supershifted with an antibody are bracketed. (B) Nuclear extracts were prepared from HeLa cells infected with a virus that expresses the WT E4-6/7 protein (lanes 1 to 4 and 9 to 12) or the F-125-P mutant E4-6/7 protein (lanes 5 to 8 and 13 to 16). The extracts were incubated in binding reaction mixtures with a DNA probe containing the E2F double site from the E2a promoter (lanes 1 to 8) or a single E2F binding site (lanes 9 to 16), and protein-DNA complexes were resolved by gel mobility shift assay. Odd-numbered lanes are binding reactions without antibody while even-numbered lanes are binding reactions that contained M45 (indicated above each lane by − and + mAb45). Lanes 1, 2, 5, 6, 9, 10, 13, and 14 are binding reactions without specific competitor DNA added (indicated above the lanes by − competitor). Lanes 3, 4, 7, 8, 11, 12, 15, and 16 are binding reactions to which a 5,000-fold molar excess of unlabeled competitor DNA (same as the double-site DNA fragment) was added after a 1-h binding reaction and

activity of the F-125-P mutant in vivo, we inserted the WT E4-6/7 or F-125-P coding sequences into an E1A-negative and E4-6/7-negative Ad vector. E4-6/7-expressing viruses, Ad-CMV-6/7-WT and Ad-CMV-6/7-F125P, were tested for E2 transactivation by coinfection of HeLa cells with a reporter virus, E2-LS-CAT, which contains the bacterial CAT gene under the control of the E2a promoter and carries a linker scanning mutation in the ATF binding site (40) in order to correlate measured CAT activity with transcriptional effects connected to the E2F sites. These data are presented in Fig. 3A. Single infection with the E2-LS-CAT virus yielded minimal CAT activity, as expected in the absence of both the E1A and E4-6/7 products. Coinfection with a WT virus that expressed both E1A and E4-6/7 (Ad5) induced CAT expression ~80-fold above the basal level seen with the reporter virus alone. Coinfection with a virus that expressed the E1A, but not E4-6/7, products (*dl*356) increased CAT expression ~90-fold. A similar level of induction was observed with Ad-CMV-6/7-WT, a virus which expressed E4-6/7 but not the E1A proteins. The mutant E4-6/7 protein F-125-P did not stimulate CAT expression over basal activity (Ad-CMV-6/7-F125P). To demonstrate that the F-125-P and E4-6/7-WT proteins were expressed at comparable levels, aliquots of the cellular extracts were subjected to immunoprecipitation and subsequent Western blot analysis with an E4-ORF7-specific monoclonal antibody (Fig. 3B). The Ad5 E4-6/7 protein (Ad5), which is functionally indistinguishable from the Ad2 form, migrates at ~17 kDa on SDS-PAGE, while the Ad2 protein (Ad-CMV-6/7-WT) has an apparent molecular mass of ~25 kDa. The F-125-P mutant protein (Ad-CMV-6/7-F125P) accumulated to a level comparable to that of the WT product. The truncated *dl*356 E4-6/7 protein was not detected because it lacks the epitope for the antibody used here. Therefore, the failure of F-125-P to transactivate the E2a promoter indicates that the formation of a complex containing E2F, E4-6/7-F125P, and one E2F binding site in the E2a promoter is not sufficient for transcriptional activation. Rather, transactivation is directly correlated with the induction of cooperative and stable E2F binding to the E2a promoter.

The F-125-P defect in stable double-site complex formation can be rescued by a divalent antibody or a covalently attached protein dimerization motif. To test the in vitro binding behavior of the virus-produced F-125-P protein, nuclear extracts from Ad-CMV-6/7-WT or from Ad-CMV-6/7-F125P-infected cells were used in gel mobility shift assays with the E2a promoter double-site probe (Fig. 4A). As observed with the bacterially expressed E4-6/7 proteins, Ad-CMV-6/7-WT-infected nuclear extract showed induction of the E2F double-site complex (lanes 1 versus 9), while Ad-CMV-6/7-F125P-infected extract only generated the single-site complex (lane 5). We performed antibody supershift experiments to confirm the presence of the F-125-P protein in the more slowly migrating band in lane 5 compared with the two E2F complexes seen with uninfected extracts (lane 9). Surprisingly, the inclusion of an E4-6/7-specific MAb (M45; epitope in the ORF6 segment of E4-6/7 [data not shown]) in the F-125-P binding reaction resulted in a supershifted complex that was not discernible in mobility and intensity from the supershift observed with the MAb and E4-6/7-WT-infected extracts (lanes 2 and 6). The same effect was observed with a MAb (M41) which recognizes a different epitope in the ORF6 segment of E4-6/7 (lanes 4 and 8). Monovalent Fab fragments of M45 did not generate this complex with F-125-P-containing extracts, but rather only slightly retarded the mobility of the E2F–F-125-P complex (lane 7).

These data suggested that the divalent MAbs, but not the monovalent Fab fragment, may have substituted for the function lost with the F-125-P mutant protein. One property of the E2F–E4-6/7 complex at the E2a promoter is its stability against challenge by a competitor DNA (21, 49). In the off-rate experiment whose results are shown in Fig. 4B, protein complexes preformed on double- or single-site probes were challenged with an excess of unlabeled double-site oligonucleotide. E4-6/7-WT-containing complexes on the double site were stable to a 5,000-fold molar excess of unlabeled DNA in the presence or absence of antibody (lanes 1 to 4). In contrast, the F-125-P complex on the double site was unstable and dissociated in the presence of the competitor oligonucleotide (lanes 5 versus 7). In contrast, the complex formed with the F-125-P protein and M45 was stable (lanes 6 versus 8). The stabilizing effect exerted by the antibody on the E2F–F-125 protein-DNA complex was dependent on the integrity of the two E2F binding sites in the E2a promoter since complexes formed with the WT or F-125-P proteins and E2F on a single site were unstable in the presence or absence of antibody (lanes 9 to 16).

To investigate the possibility that F-125-P is defective for protein-protein interaction that is required for E2F dimerization at the double site and which was artificially provided through the divalent nature of the antibody interaction, we attempted to rescue the ability of the F-125-P mutant protein to induce E2F binding by engineering a heterologous protein dimerization domain into the mutant protein. The C/EBP leucine zipper was fused to the N terminus of both E4-6/

for which incubation was continued for an additional hour (indicated above the lanes by + competitor). The positions of free E2F, the E2F–E4-6/7 single-site complex, and the induced E2F double-site complex are indicated on the left. The positions of complexes supershifted by M45 are indicated by brackets on the left and right. (C) WT and mutant E4-6/7 proteins and E4-6/7 proteins fused to the C/EBP leucine zipper were synthesized by in vitro translation with a wheat germ translation extract and added to binding reactions that contained cytoplasmic extract from *dl*356-infected HeLa cells and an E2F double-site probe from the E2a promoter region. Protein-DNA complexes were resolved by gel mobility shift assay. Binding reaction mixtures contained HeLa cell extract alone (lane 1), extract plus increasing amounts (2 and 4 μl) of wheat germ extract lacking RNA (− RNA, lanes 2 and 3), extract plus increasing amounts (0.5 to 4 μl) of WT E4-6/7 protein (+ 6/7 WT, lanes 4 to 7), extract plus increasing amounts (0.5 to 4 μl) of E4-6/7 F-125-P mutant protein (+ 6/7 F125P, lanes 8 to 11), and increasing amounts (0.5 to 4 μl) of WT and F-125-P mutant proteins fused to the C/EBP leucine zipper (+ ZIP 6/7 WT, lanes 12 to 15, and + ZIP 6/7 F125P, lanes 16 to 19). The positions of free E2F, the E2F–E4-6/7 single-site complex, and the induced E2F double-site complex are indicated on the left. (D) Nuclear extract from WT Ad5-infected HeLa cells was incubated with an E2F double-site probe from the E2a promoter region. Protein-DNA complexes were resolved by gel mobility shift assay. The extract was incubated alone (lane 1) or with increasing concentrations of M41 (lanes 2 to 6; note that the antibody was diluted for addition to reaction mixtures in lanes 2 to 4 but is indicated as fractions of microliters for comparison with higher concentrations). The reaction mixture in lane 7 contained 1 μl of M41 plus 4 μl of a nonrelated MAb (αEBNA) of the same protein concentration. Lane 8 contained only 4 μl of the αEBNA MAb. The position of the induced E2F complex is indicated on the right.

7-WT and F-125-P products, and the fusion proteins were expressed in vitro with a wheat germ translation extract and assayed for E2F induction. In the experiment whose results are shown in Fig. 4C, increasing amounts of in vitro-translated proteins were titrated into double-site binding reactions with *dl*356-infected cytoplasmic extract. Both E4-6/7-WT (lanes 4 to 7) and the ZIP-E4-6/7-WT fusion protein (lanes 12 to 15) reconstituted the E2F infection-specific complex. In vitro-translated E4-6/7-F125P formed only the single-site complex as expected (lanes 8 to 11), whereas the ZIP-E4-6/7-F125P protein restored the double-site complex (lanes 16 to 19). The E2F complex induced by the ZIP-E4-6/7-F125P fusion protein was stable to oligonucleotide challenge in off-rate analyses, while the F-125-P–E2F complex was not (data not shown).

Further indication that two E4-6/7 proteins are present in the double-site complex with E2F came from titration experiments with Ad-infected nuclear extracts and increasing concentrations of E4-6/7 MAb41 (Fig. 4D). As the concentration of the MAb in the binding reaction mixture was increased, a supershifted complex (lanes 3 and 4) was chased into a second distinct band of slower mobility (lanes 5 and 6). This effect was specific for the anti-E4-6/7 MAb (lanes 7 versus 8). The slowest-mobility supershift likely represents a second antibody molecule recognizing a second E4-6/7 epitope in the E2F–E4-6/7 protein-DNA complex. Collectively, these data indicate that more than one E4-6/7 molecule is necessary to form the induced E2F complex at the E2a promoter and that the F-125-P mutant protein likely is defective for dimerization.

E2F and E4-6/7 form a stable complex in solution. Extract clearing experiments with E4-6/7-specific antiserum (27) and cofractionation of E4-6/7 with E2F DNA binding activity (37) have indicated that the protein components of E2F and the E4-6/7 protein participate in complexes when no exogenous binding site is added. We devised an immunoprecipitation assay coupled with mobility shift analysis in order to assess the properties of these interactions and to attempt to visualize components of this protein association. HeLa cells were infected with Ad5 (E4-6/7 positive) or *dl*356 (E4-6/7 negative) or left uninfected and pulse-labeled for 2 h with ^{35}S-Translabel at 4 h postinfection. Nuclear extracts were prepared and immunoprecipitated with the E4-6/7-specific MAb45 under in vitro binding conditions but without the addition of E2F-specific binding sites. The immunoprecipitates were washed with buffers containing 1 M salt and 0.1% SDS, and precipitated, labeled proteins were eluted from the antibody-protein A complex with the cognate P2 peptide and assayed for in vitro DNA binding activity (Fig. 5A) or analyzed by SDS-PAGE (Fig. 5B). E2F DNA binding activity was recovered in the immunoprecipitates in a manner that was dependent on the presence of functional E4-6/7 (Fig. 5A, lanes 7 to 9). E2F DNA binding activity was also recovered when recombinant E4-6/7 and uninfected extracts were incubated and immunoprecipitated (data not shown). The recovered complex in the P2 eluate from Ad-infected extract was authentic since it was supershifted with a second E4-6/7-specific antibody (MAb41) which is not blocked by the peptide in the binding reaction (Fig. 5A, lane 10). Two specific and several enriched protein species were evident by SDS-PAGE in immunoprecipitates from WT-infected extracts compared with *dl*356-infected or uninfected extracts (Fig. 5B, lanes 1 to 3, circles and bracket). The mobility of these proteins corresponds with the expected sizes of E2F-related products (23, 28, 29, 51), and the presence of several species is consistent with the demonstration that more than

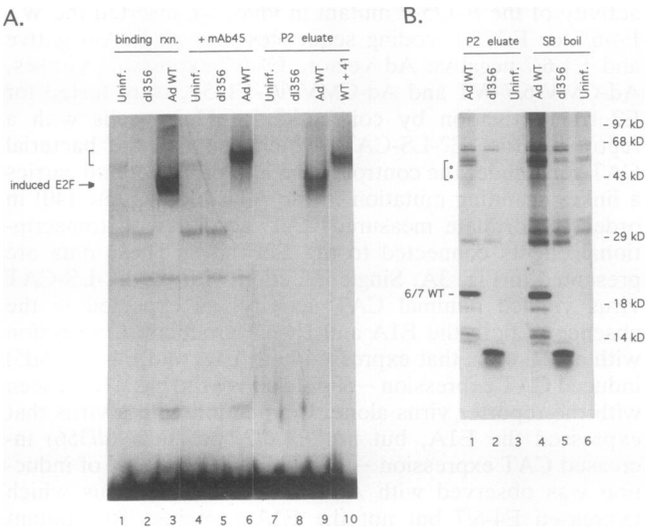


FIG. 5. E2F and E4-6/7 form a stable complex in solution. Binding reactions (10×) were performed with ^{35}S-labeled nuclear extracts from uninfected (Uninf.), WT Ad5-infected (Ad WT), and *dl*356-infected (dl356) HeLa cells and the E2F double-site probe from the E2a promoter region. Following a 1-h incubation, an aliquot was removed and analyzed by gel mobility shift assay (panel A, lanes 1 to 3). M45 was added to each binding reaction mixture, incubation was continued for 1 h, and a second aliquot was removed for mobility shift analysis (panel A, lanes 4 to 6). The remainder of each binding reaction mixture was immunoprecipitated with protein A-Sepharose and washed with RIPA and high salt, and E2F–E4-6/7 complexes were eluted with the cognate peptide for M45 (P2; see Materials and Methods). An aliquot of each eluate was analyzed by mobility shift analysis (panel A, lanes 7 to 9). In panel A, lane 10, M41 was added to an aliquot of the Ad WT P2 eluate and incubated prior to mobility shift assay. The induced E2F complex is indicated in panel A on the left, and complexes supershifted by the MAbs are bracketed. (B) Equal aliquots of the P2 eluate from each sample were analyzed by SDS-PAGE (lanes 1 to 3). In addition, the protein A-Sepharose beads were boiled after peptide elution in sample buffer and the released material was analyzed by SDS-PAGE (lanes 4 to 6). The WT E4-6/7 protein is indicated on the left; the faster-migrating product in lane 2 represents the *dl*356 truncated E4-6/7 polypeptide. The positions of molecular mass markers are indicated on the right. The specific and enriched proteins present in immunoprecipitates of Ad WT extracts (lanes 1 and 4) and described in the text are indicated by open circles and a bracket, respectively, on the left.

one E2F activity is required to form the Ad infection-specific E2F–E4-6/7 complex (see below). These results show that the interaction of E4-6/7 and E2F is independent of specific E2F recognition sites and is stable to salt and detergent conditions which obstruct E2F DNA binding (data not shown). This suggests that the fast off-rate of the single-site E2F–E4-6/7 complex in the gel shift assay is a function of weak protein-DNA interactions and likely does not reflect an intrinsic instability of the solution complexes involving E2F and E4-6/7.

At least two cellular components are required for formation of the induced E2F double-site complex. Recent reports have described the cloning of an Rb-binding protein that exhibits the characteristic DNA binding properties of E2F (23, 29, 51). Indirect evidence suggests that this clone, referred to as E2F-1, participates in the E4-6/7–E2F complex. First, a GST-6/7 fusion protein can interact with a protein in the E2F-1 molecular weight range which is recognized by anti-

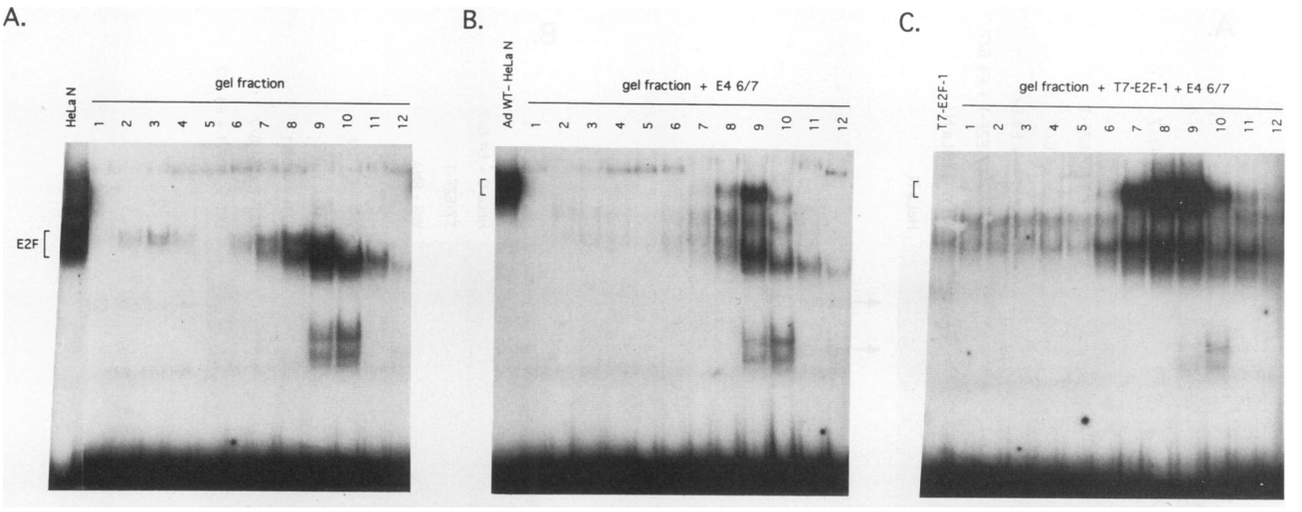
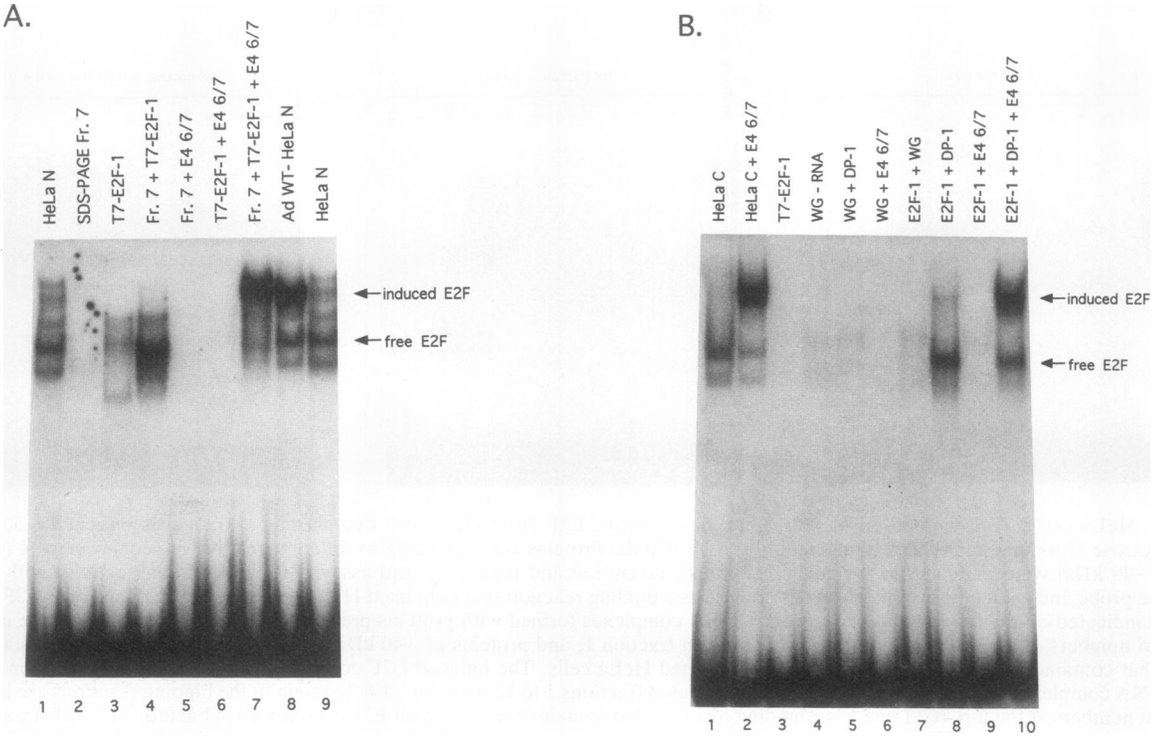


FIG. 6. HeLa cell E2F is composed of more than one protein. E2F from HeLa cell cytoplasmic extract was enriched ~50-fold by heparin-agarose chromatography and fractionated by SDS-PAGE. Proteins corresponding to different relative molecular masses (between ~70 and ~40 kDa) were eluted from the gel, precipitated, denatured and renatured, and assayed for E2F binding activity with an E2F double-site probe and gel mobility shift assay. (A) Lane 1 is a binding reaction that contained HeLa nuclear extract alone. Free E2F binding activity is indicated on the left by a bracket. Protein-DNA complexes formed with proteins present in eluted fractions 1 to 12 are indicated by fraction numbers at the top (proteins at ~70 kDa are in fraction 1, and proteins at ~40 kDa are in fraction 12). (B) Lane 1 is a binding reaction that contained nuclear extract from Ad WT-infected HeLa cells. The induced E2F complex is indicated by a bracket on the left. Protein-DNA complexes formed with proteins present in eluted fractions 1 to 12 with the E4-6/7 protein in the binding reactions are indicated by fraction numbers at the top. (C) Lane 1 is a binding reaction that contained recombinant E2F-1 produced in bacteria (T7-E2F-1); a doublet of binding activity is evident. Protein-DNA complexes formed with recombinant E2F-1, proteins present in the eluted fractions 1 to 12 as indicated at the top, and E4-6/7 protein in the binding reactions. The mobility of induced E2F activity is indicated by a bracket at the left.

E2F-1 antiserum. Second, anti-E2F-1 antibodies precipitate a DNA binding activity from HeLa cell extracts that binds to the E2a promoter and is induced by E4-6/7 (29). In our work, however, bacterially expressed E2F-1 or E2F-1 translated in vitro failed to interact with E4-6/7 in gel shift analyses or in coimmunoprecipitation assays. This could be due to missing modifications of recombinant E2F-1 or to E4-6/7 interaction with an E2F-1-related, but distinct, protein which cross-reacts with the E2F-1-specific antiserum. The available data (28) also suggest the possibility that an additional cellular component(s) may be necessary to form an E2F-1- and E4-6/7-containing complex. To test this third hypothesis, HeLa cytoplasmic extract was enriched ~50-fold for E2F activity by passage over a heparin-agarose column and fractionated by preparative SDS-PAGE. Proteins from different gel slices corresponding in molecular mass to between ~40 and ~70 kDa were eluted, renatured, and assayed for binding to the E2a promoter either alone (Fig. 6A), in conjunction with bacterially produced GST-E4-6/7 (Fig. 6B), or in combination with E4-6/7 and bacterially produced recombinant E2F-1 (Fig. 6C). Numerous binding activities, particularly in fractions 7 to 11, bound to the double-site probe to generate complexes similar in mobility to those observed with HeLa nuclear extract (Fig. 6A). Fraction 9 displayed the strongest E2F-like activity by itself (Fig. 6A) and the highest level of induction of the double-site E2F–E4-6/7 complex when assayed in a binding reaction with E4-6/7 (Fig. 6B). Proteins in fractions 8 and 10 were also capable of interaction with E4-6/7 to form a double-site complex, whereas none of the other fractions in this experiment were induced by E4-6/7 (Fig. 6B). This picture changed significantly when recombinant E2F-1 was included in the binding reactions together with the eluted fractions and E4-6/7 (Fig. 6C). Binding was greatly stimulated not only in fractions 8 through 10, but also in fraction 7, which did not form the double-site complex in the absence of recombinant E2F-1 (compare Fig. 6B with C). Additional mixing experiments (Fig. 7A) show that proteins in fraction 7 (lane 2) or recombinant E2F-1 alone (lane 3) bound poorly to the double-site probe. Mixing of fraction 7 and E2F-1, however, restored a major complex equivalent in mobility to the upper form of E2F observed with HeLa nuclear extract (lanes 1 and 4). These findings are in agreement with the results reported by Huber and coworkers (28) and show that optimal DNA binding activity is dependent on heterodimerization of E2F-1 and a distinct component(s). Moreover, fraction 7 exemplifies the requirement for a cellular component(s) in addition to E2F-1 for double-site complex formation. Neither fraction 7 nor E2F-1 alone interacted with E4-6/7 on the double site (lanes 5 and 6), whereas the combination of fraction 7, E2F-1, and E4-6/7 resulted in the formation of a complex with mobility similar to that of the stable complex seen with Ad-infected nuclear extract (lanes 7 and 8). The complex observed in lane 7 has a slightly retarded mobility in comparison with the Ad-induced complex (lane 8) because a GST-E4-6/7 fusion protein was used in this assay.

E2F-1, DP-1, and E4-6/7 can reconstitute the double-site complex in vitro. DP-1 was isolated from an F9 cell cDNA library following site-specific DNA affinity purification of the DRTF-1 binding activity, which seems to be identical or closely related to the HeLa cell E2F activity (16). We tested whether DP-1 could interact with E4-6/7 in the absence of other proteins or in conjunction with recombinant E2F-1 (Fig. 7B). DP-1 was synthesized by in vitro translation with a wheat germ extract; a major species of ~50 kDa was evident following translation by SDS-PAGE (data not shown). When in vitro-translated DP-1 was incubated with the double-site probe, two minor protein-DNA complexes



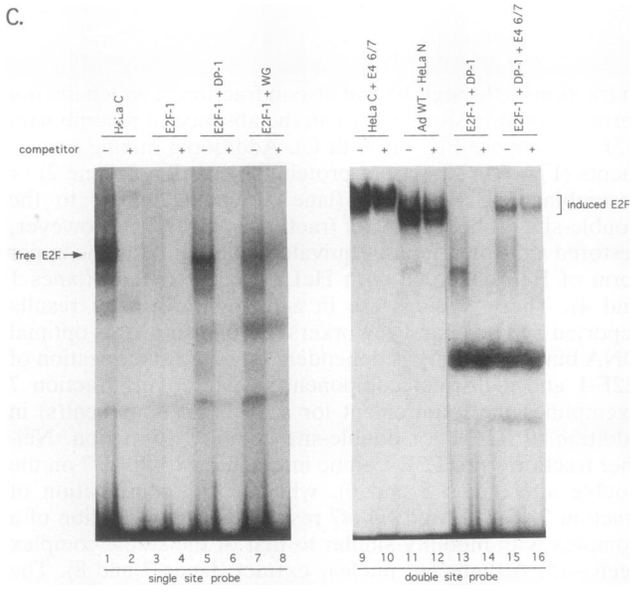


FIG. 7. E2F-1, DP-1, and E4-6/7 are sufficient to form the induced E2F double-site complex. (A) Binding reactions that contained proteins present in SDS-PAGE fraction 7, recombinant E2F-1, and E4-6/7 were performed and analyzed by gel mobility shift assay. Lanes 1 and 8 are binding reactions that contained HeLa nuclear extract alone. Lanes 2 and 3 are binding reactions that contained SDS-PAGE fraction 7 alone or E2F-1 alone, respectively. Lane 4 is a binding reaction that contained fraction 7 and E2F-1. Lanes 5 and 6 are binding reactions that contained fraction 7 plus E4-6/7 or E2F-1 plus E4-6/7, respectively. Lane 7 is a binding reaction that contained fraction 7, E2F-1, and E4-6/7. Lane 8 is a binding reaction that contained nuclear extract from Ad WT-infected HeLa cells. The positions of the free E2F and induced E2F complexes are indicated on the right. (B) Binding reactions that contained E2F-1, DP-1, and E4-6/7 were performed and analyzed by gel mobility shift analysis. Lane 1 is a binding reaction that contained uninfected HeLa cytoplasmic extract. Lane 2 is a binding reaction that contained HeLa cytoplasmic extract plus E4-6/7 protein. Lane 3 is a binding reaction that contained recombinant E2F-1 alone. Lanes 4 to 6 are binding reactions that contained 4 μl of wheat germ translation extract programmed with no RNA (WG-RNA, lane 4), DP-1 RNA (WG + DP-1, lane 5), or E4-6/7 RNA (WG + E4 6/7, lane 6). Lane 7 is a binding reaction that contained recombinant E2F-1 plus unprogrammed wheat germ extract. Lane 8 is a binding reaction that contained recombinant E2F-1 plus DP-1 synthesized in vitro. Lane 9 is a binding reaction that contained E2F-1 plus E4-6/7. Lane 10 is a binding reaction that contained E2F-1, DP-1, and E4-6/7. The positions of free E2F and induced E2F are indicated on the right. (C) Binding reactions that contained E2F-1, DP-1, and E4-6/7 were performed and analyzed by gel mobility shift analysis. Reactions in lanes 1 to 8 contained a single E2F binding site, and reactions in lanes 9 to 16 contained the E2F double site from the E2a promoter. Lanes 1 and 2 are binding reactions that contained uninfected HeLa cell extract (HeLa C). Lanes 3 to 8 and 13 to 16 are binding reactions that contained recombinant E2F-1 alone (E2F-1, lanes 3 and 4); E2F-1 with 4 μl of wheat germ translation extract programmed with DP-1 RNA (E2F-1 + DP-1, lanes 5, 6, 13, and 14); E2F-1 plus wheat germ extract with no RNA (E2F-1 + WG, lanes 7 and 8); or E2F-1, DP-1, and E4-6/7 protein expressed in wheat germ extract (lanes 15 and 16). Lanes 9 and 10 are binding reactions that contained uninfected HeLa cell cytoplasmic extract and bacterially expressed E4-6/7 protein (HeLa C + E4 6/7). Lanes 11 and 12 are binding reactions that contained nuclear extract from Ad WT-infected HeLa cells (Ad WT-HeLa N). Odd-numbered lanes are binding reactions without specific competitor DNA added (indicated by − above each lane). Even-numbered lanes are binding reactions to which a 5,000-fold molar excess of unlabeled competitor DNA (same as the double-site DNA fragment) was added after a 1-h binding reaction and for which incubation was continued for an additional hour (indicated by + above each lane). The position of free E2F is indicated on the left. The positions of the induced E2F double-site complexes are indicated on the right by a bracket. The material evident in lanes 13 to 16 near the middle of the gel represents [^{35}S]methionine present in the wheat germ translations.

that were also evident with unprogrammed wheat germ extract were observed (Fig. 7B, lanes 4 and 5). This suggests no or very weak affinity of in vitro-translated DP-1 for the E2F sites analogous to the results with recombinant E2F-1 (lane 3). However, when recombinant E2F-1 was mixed with in vitro-translated DP-1, a major complex that was indistinguishable from the upper E2F complex observed with uninfected cellular extract was observed (lanes 1 versus 8). The small amount of double-site complex produced with recombinant E2F-1 and DP-1 was greatly induced upon addition of E4-6/7 (lanes 8 and 10). E4-6/7 alone (lane 6) or in combination with E2F-1 (lane 9) or DP-1 (data not shown) did not specifically interact with the probe. Complexes formed by mixing E2F-1 and DP-1 on single or double E2F binding sites were unstable when challenged with cold competitor DNA like the endogenous E2F activity in uninfected HeLa cell extract (Fig. 7C, lanes 1 to 8 and 13 to 14). In contrast, the E2F-1–DP-1 complex induced by the E4-6/7 protein on the double-site probe was stable to competitor challenge like the complex formed with Ad-infected HeLa cell extract or uninfected HeLa cell extract with exogenous E4-6/7 protein added to the binding reaction mixture (Fig. 7C, lanes 9 to 12 and 15 to 16). From these data, we conclude that E2F-1, DP-1, and E4-6/7 are sufficient to form a double-site complex at the E2a promoter with the properties of the double-site complex induced by Ad infection.

DISCUSSION

Ad infection-specific protein complexes formed at single and inverted repeat E2F recognition sites and their correlation with function. When the binding of E2F to Ad promoters during an Ad infection is analyzed in vitro, two distinct infection-specific complexes are observed with a gel mobility shift assay depending on the presence of a single E2F recognition site or the two inverted E2F binding sites in the E2a promoter. The E4-6/7 protein is involved in these complexes (27, 37, 41, 49). In this study, we describe two point mutants in the E4-6/7 protein (Fig. 1, F-125-P and F-125-A) which retain the ability to interact with E2F at a single E2F binding site but are defective for formation of the Ad-induced E2F complex at the double site in the E2a promoter (Fig. 2). The results obtained with these mutants are in agreement with a report by Neill and Nevins (42) in which internal deletions in E4-6/7 between amino acids 104 and 115 or 115 and 129 generated mutants with the same phenotype. The phenotype of the F-125 mutants indicates that the generation of an induced E2F double-site complex requires an activity of the E4-6/7 polypeptide in addition to or entirely distinct from features that allow E4-6/7 to interact with E2F at a single site. It is not clear whether E2F–E4-6/7 single-site complex formation is a required step in the pathway for cooperative binding of E2F to the E2a promoter. Several point mutants (T-133-Q, G-141-L, V-149-N, and R-101-A) were capable of double-site complex formation (Fig. 2B) but did not generate a single-site complex in the gel shift assay (Fig. 2A). Thus, the interaction of E4-6/7 with E2F at a single site may reflect a different function in comparison with induction of E2F binding at the double site in the E2a promoter. Perhaps, a more likely explanation of the phenotype of these particular mutants is that they have decreased affinity for E2F that results in dissociation under conditions of electrophoresis during the gel shift assay, whereas once trapped in a stable E2F double-site complex, the mutant proteins are resistant to dissociation.

The phenotype of mutant F-125-P enabled us to probe for possible transcriptional function of single-site versus double-site E2F complexes at the E2a promoter. E4-6/7 carrying the F-125-P substitution and expressed in vivo from a viral genome did not transactivate the E2a promoter of a reporter virus in a HeLa cell coinfection assay (Fig. 3). Thus, the single-site complex formed with F-125-P at the E2a promoter in vitro is not sufficient for transcriptional stimulation by E4-6/7 in vivo. This analysis is in agreement with a characterization of the functional *cis*-acting elements in the E2a promoter performed in HeLa cells with virus vectors (36). Mutation of either E2F site significantly reduced transcriptional activation of the E2a promoter in the presence of E4 (36). Our results, however, differ from those presented by Neill and Nevins (42), who observed transactivation of the E2a promoter by E4-6/7 mutants that were deficient in the induction of stable E2F binding to the double site. This difference could reflect the assays used (virus infection versus transient expression assays), the cell lines used in the analyses (HeLa versus Vero), or the reporter vectors used (LS-ATF-E2-CAT versus WT-E2-CAT). The cell line used may be particularly relevant since E2F is found in different protein complexes in different cell lines; HeLa cells have a relative abundance of free E2F compared with other cells and may, in the absence of E1A expression, most closely mimic conditions of Ad infection in which the E1A products disrupt E2F-cellular protein complexes to release free E2F which is then available for interaction with E4-6/7 (1, 49). The finding that transcriptional activation does not correlate in our assays with E2F binding to a single site in the E2a promoter cannot be extended to the function of E2F when bound to a single recognition site in general. Functional E2F sites are present in the E1A enhancer region (32) where no cooperative E2F complex is formed because of the larger spacing of the two sites (21), and single functional E2F sites have been identified in cellular promoters such as the dihydrofolate reductase promoter (7). E2F-dependent transcriptional stimulation may involve neighboring factor binding sites or vary with the type and status of the host cell. Indeed, the single E2F site of the dihydrofolate reductase promoter is sufficient for growth-regulated promoter activity (54).

Dimerization of E4-6/7 appears to be involved in the mechanism of E2F induction at the E2a promoter. Combined evidence from the following experiments suggests the presence of at least two E4-6/7 polypeptides in the E2F–E4-6/7 double-site complex and the role of E4-6/7 dimerization in E2F induction. First, addition of E4-6/7-specific divalent MAbs to binding reaction mixtures containing E2F, the F-125-P mutant protein, and the double-site probe generated a stable, supershifted complex of mobility and quantity equal to those observed with WT E4-6/7 (Fig. 4A and B). Since F-125-P did not form the double-site complex in the absence of antibody or with a monovalent Fab fragment, two possibilities can be invoked to explain this result. The antibody may interact with two unstable E2F–E4-6/7 complexes on the same double-site probe fragment to stabilize their interaction at the inverted repeat. Alternatively, the antibody may bridge two double-site probe fragments each occupied at a single E2F site. It is unlikely that the complex described in the second scenario would be stable since supershifted complexes formed on probe fragments containing a single binding site had fast off-rates (Fig. 4B). Fab fragments did not induce or stabilize F-125-P-containing complexes, arguing that divalency of the intact antibody is required and that the interaction of one E4-6/7 epitope with the antigen recognition surface of the antibody is not sufficient for the observed effect. Secondly, the C/EBP leucine zipper co-

valently attached to the F-125-P protein restored the ability of the mutant product to induce stable E2F binding to the double site (Fig. 4C). Leucine zippers are well-characterized dimerization elements forming a parallel α-helical coiled-coil structure (45). The restoration of double-site complex formation to the F-125-P protein by the leucine zipper is likely mediated by dimerization of two ZIP-F125P fusion proteins. However, the interaction of one fusion protein with the leucine zipper of an E2F component is not entirely inconceivable, although the stability of the interactions between E2F components in 4 M urea (data not shown) and high salt and detergent (Fig. 5) argues against this possibility and for the presence of stable E2F dimers in cellular extracts. The final indication for the presence of two E4-6/7 molecules in the induced E2F double-site complex comes from binding reaction mixtures containing Ad-infected cell extract and increasing concentrations of an E4-6/7-specific MAb (Fig. 4D) in which a supershifted complex observed at limiting antibody concentrations was replaced by a second, more slowly migrating complex. The simplest explanation for the second supershift is the presence of two E4-6/7 epitopes in the induced E2F complex. The likely presence of more than one E4-6/7 protein in the E2a promoter complex could reflect an intrinsic property of the E4-6/7 protein to homodimerize, although we have not been able to establish evidence for the presence of homodimeric or multimeric E4-6/7 complexes in solution. E4-6/7 protein synthesized by in vitro translation with wheat germ extract, which is not associated with E2F, migrates in gel filtration at the size expected for a monomeric polypeptide and retains E2F induction activity (unpublished observations). E4-6/7 may not interact with itself in solution but may require assembly of the E2F–E4-6/7 complex at the E2a promoter in order to dimerize. Alternatively, a bridging dimerization surface may be provided by another protein in the protein-DNA complex.

Without information on the protein structure of E4-6/7, the amino acid substitutions and the nature of the resulting phenotypes can only be correlated to structural motifs predicted by computer analysis. The two segments of E4-6/7 that are required for interaction with E2F at a single site do not display any notable predicted structure. It is interesting that two distinct segments of E4-6/7 are involved since this is reminiscent of the pocket proteins such as Rb and p107 that interact directly with E2F and that have two distinct regions that are required for interaction (2, 3, 10, 11). Possibly, the distinct segments interact independently with the different components of an E2F heterodimer. The predicted α-helices in the E4-6/7 protein, separated by a potential loop, have been speculated to be a dimerization motif in this protein (42) by analogy to the helix-loop-helix family of dimeric transcription factors. The results that we obtained with the E4-6/7 substitution mutants do not correlate with the results of similar mutations introduced into a notable helix-loop-helix transcription factor, myoD (13). Specifically, proline substitution in either α-helix in myoD disrupted dimerization while no direct correlation in this regard was found with E4-6/7 by analyzing proline substitutions or comparing proline versus alanine substitutions (Q-105-P versus Q-105-A, D-121-P versus D-121-A, and F-125-P versus F-125-A). Additionally, insertion of six alanine residues in the loop region of myoD did not decrease dimerization while this substitution in E4-6/7 disrupted E2F induction activity. Thus, the E4-6/7 protein does not appear to dimerize by a helix-loop-helix motif.

E2F components in the double-site complex. What are the components of the Ad-induced complex at the E2a promoter in addition to the E2F recognition sites and E4-6/7? Crude fractionation of HeLa cell extracts by SDS-PAGE separated a fraction with very weak E2F DNA binding activity (Fig. 6, fraction 7) from the major peak of E2F activity. This fraction was not inducible by E4-6/7 in contrast to the peak E2F fractions. Recombinant E2F-1 in our work binds weakly to E2F sites and does not interact with E4-6/7. Mixing of E2F-1 with the proteins in gel fraction 7 stimulated E2F binding activity considerably and allowed formation of the double-site complex containing E4-6/7 (Fig. 7A). These data are in agreement with a recent report that purified E2F consists of two groups of proteins which need to heterodimerize to efficiently bind DNA (28). Recombinant E2F-1 and DP-1 recently have been shown to form stable heterodimers (4, 24). Here we show that at least two distinct cellular components are required for double-site complex formation with E4-6/7, one of which can be substituted by E2F-1. In addition, cooperative complex formation can be reconstituted entirely from recombinant components. When combined, recombinant E2F-1 and DP-1 were significantly enhanced in binding affinity for E2F sites and generated the double-site complex in the presence, and to a much lesser extent in the absence, of E4-6/7 (Fig. 7B). This indicates that two different E2F family members are necessary for double-site complex formation. Additionally, the stability of the complex containing the E4 6/7 protein, E2F-1, and DP-1 on the double site was comparable to that of the Ad-induced E2F activity (Fig. 7C). At this time, we do not know whether E4-6/7 has specificity for particular E2F heterodimers or what combinations of E2F components constitute functional activity in vivo to mediate E4 transactivation.

On the basis of the evidence that E2F binds to a single binding site as a heterodimer and that at least two E4-6/7 proteins are present in the double-site complex, the simplest hypothesis for double-site complex stoichiometry is that two E2F heterodimers interact with two E4-6/7 proteins at the E2a promoter. E2F is associated with E4-6/7 in the absence of DNA recognition sites in vivo as demonstrated by coimmunoprecipitation experiments. These solution complexes are stable under stringent salt and detergent conditions. Since E4-6/7 does not appear to dimerize in solution, E4-6/7 probably does not function by bringing together two E2F heterodimers before binding to the E2a promoter. At the E2a promoter, E4-6/7 could provide a direct dimerization function or could induce an interaction surface in E2F to facilitate binding of a second E2F heterodimer. The availability of recombinant components should help address this question.

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